Can lipid removal affect interpretation of resource partitioning from stable isotopes in Southern Ocean pteropods?

Christine K. Weldrick[1,2*], Rowan Trebilco[1,2], and Kerrie M. Swadling[1,2]

[1] *Institute for Marine and Antarctic Studies, University of Tasmania, Private Bag 129, Hobart, Tasmania 7001, Australia*
[2] *Antarctic Climate and Ecosystems Cooperative Research Centre, University of Tasmania, Private Bag 80, Hobart, Tasmania 7001, Australia*

* *Correspondence to:* C. K. Weldrick, Institute for Marine and Antarctic Studies, University of Tasmania, Private Bag 129, Hobart, Tasmania 7001, Australia.
Email: christine.weldrick@utas.edu.au

Keywords:

Lipid normalization, lipid correction, $\delta^{13}C$ and $\delta^{15}N$, niche dispersion metrics, polar, zooplankton

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/rcm.8384

Abstract

RATIONALE: Stable isotopes analysis (SIA) is a powerful tool to estimate dietary links between polar zooplankton. However, the presence of highly variable ^{12}C-rich lipids may skew estimations as they are depleted in ^{13}C relative to proteins and carbohydrates, consequently masking carbon signals from food sources. Lipid effects on pteropod-specific values requires examining, since accounting for lipids is rarely conducted among the few existing pteropod-related SIA studies. It is currently unclear whether lipid correction is necessary prior to SIA of pteropods.

METHODS: Whole bodies of three species of pteropods (*Clio pyramidata* f. *sulcata*, *Clione limacina antarctica*, and *Spongiobranchaea australis*) sampled from the Southern Ocean were lipid-extracted chemically to test the effects on $\delta^{13}C$ and $\delta^{15}N$ values (n=38 individuals in total). We determined the average change in $\delta^{13}C$ values for each treatment, and compared this offset with those from published normalization models. We tested lipid correction effects on isotopic niche dispersion metrics to compare interpretations surrounding food web dynamics.

RESULTS: Pteropods with lipids removed had $\delta^{13}C$ values up to 4.5‰ higher than bulk samples. However, lipid extraction also produced higher $\delta^{15}N$ values than bulk samples. Isotopic niche overlaps between untreated pteropods and their potential food sources were significantly different from overlaps generated between lipid-corrected pteropods and their potential food sources. Data converted using several published normalization models did not reveal significant differences among various calculated niche metrics, including standard ellipse and total area.

CONCLUSIONS: We recommend accounting for lipids via chemical extraction or mathematical normalization before applying SIA to calculate ecological niche metrics, particularly for organisms with moderate to high lipid content such as polar pteropods. Failure to account for lipids may result in misinterpretations of niche dimensions and overlap and, consequently, trophic interactions.

Introduction

Stable isotopes analysis (SIA) has emerged as a powerful tool for elucidating food web structure, including estimates of dietary niche breadths and degree of overlap for co-occurring species[1,2]. The most commonly used stable isotope ratios in ecological studies make use of samples that are relatively enriched in the heavier isotopes of carbon and nitrogen, with values denoted as $\delta^{13}C$ and $\delta^{15}N$, respectively[3]. $\delta^{13}C$ values vary between different basal food sources, and show relatively little fractionation (~ 1‰) between trophic levels [4,5] while $\delta^{15}N$ values typically shows greater trophic fractionation (~ 1-4 ‰)[5,6]. SIA can be an effective means for estimating the origins of consumer dietary sources and their trophic positions within food webs, but the method has limitations. Importantly, it is not known whether and how standardization (or the lack thereof) of treatment protocols for SIA[7,8] affects the interpretation of trophic patterns such as niches and their overlap.

Comparison of $\delta^{13}C$ values must take account of the potential effect of lipids, particularly when comparing samples with different lipid contents (e.g. different tissues and/or species). Lipids are depleted in ^{13}C (~ 2 to 8 ‰) relative to proteins and carbohydrates[5], and can be a potential source of error in ecological studies if left uncorrected for. The presence of lipids can lower $\delta^{13}C$ values from animal tissues possessing relatively high lipid content (i.e. lipid content > 5%, mass C:N ratios > 3.5[9]), and mask the $\delta^{13}C$ values derived from diet[10]. Zooplankton obtain lipids from food and they can also synthesize them via de novo biosynthesis[11]; these biosynthesized lipids can have different $\delta^{13}C$ values from those obtained through diet[12]. This is of particular importance when analyzing zooplankton from polar environments, such as pteropods, which have been shown to exhibit unique lipid biochemical adaptations[11,13,14] that vary both seasonally and latitudinally[15]. Among pteropods the highest lipid concentrations have been recorded for the polar gymnosome (unshelled) *Clione*

limacina, which possess fat reserves that permit them to survive long periods of food limitation during seasonally low abundances of their preferred prey, the thecosome (shelled) pteropod *Limacina* sp.[16,17].

Lipid removal is routinely achieved through chemical extraction (eg. chloroform/methanol)[18] prior to SIA, enabling the standardization of $\delta^{13}C$ values across all samples[9]. However, chemical extraction can be time-consuming, costly, may alter $\delta^{15}N$ values, and adversely affect other sample-processing steps, including acidification used to remove inorganic carbon[19]. Mathematical normalization models provide an alternative to the chemical removal of lipids from tissues; they are based on the relationship between bulk (uncorrected) C:N ratios and $\delta^{13}C$ values and have been shown to predict $\delta^{13}C$ values for other polar marine organisms[8,15]. Polar zooplankton-focused studies[8,15] have measured significant depleting effects of lipids on isotopic ratios and recommend correcting for these effects prior to further analyses. To our knowledge, no studies have tested these effects on the isotopic ratios of polar pteropods.

This study investigates the effects of lipid removal on carbon and nitrogen stable isotope ratios in pteropod assemblages from the Indian sector of the Southern Ocean. We extracted lipids from three pteropod species and sought to determine (1) whether $\delta^{13}C$ and $\delta^{15}N$ values from chemically extracted tissues differed from those from untreated tissues, (2) if mathematical normalization models were effective in correcting for lipids, and (3) if isotopic niche dispersion metrics were sensitive to chemical lipid-extraction and/or mathematical lipid correction. Few studies have employed SIA with Southern Ocean pteropods and, among them, fewer have accounted for lipids[17,20], making between-study comparisons problematic. Given the essential roles of pteropods in contributing to deep sea CO_2 sequestration[21], and

providing top down control on phytoplankton and smaller zooplankton concentrations[22], there is urgency to perform climate impact assessments on a relatively understudied organism highly sensitive to changes in ocean temperature and chemistry[23,24]. We hypothesize that lipid extraction will result in a statistically significant increase in $\delta^{13}C$ values relative to values from untreated samples that will consequently lead to significantly different outcomes in isotopic niche estimations.

Materials and Methods

Sampling of pteropods and POM

Mesozooplankton were sampled with a Rectangular Midwater Trawl 8 (RMT8) (mesh size: 4.5 mm, mouth area: 8 m^2, Nitex nylon, Australian Filter Specialists, Huntingwood, NSW, Australia), equipped with a flow meter, and towed obliquely from the surface to 200 m. The ship speed during plankton net tows was 2-2.5 knots for an average duration of 45 minutes. Thecosome (shelled) and gymnosome (naked) pteropods were counted and identified to species level. One species of thecosome, *Clio pyramidata* f. *sulcata* (hereafter *C. pyramidata)*, and two species of gymnosomes, *Clione limacina antarctica* (hereafter *C. antarctica)* and *Spongiobranchaea australis*, were separated into cryotubes, then stored in liquid nitrogen prior to analyses. Size-fractionated particulate organic matter (POM) was collected through large volume sequential filtration from the ship's underway water supply (~ 5 m depth), prescreened with an upstream 47 mm-diameter, 1 mm filter mesh for zooplankton removal, then collected onto 25 mm-diameter Sterlitech silver membrane (pore size = 1.2 µm; Sterlitech Corporation, Kent, WA, USA) and Nitex filters (pore size = 210 µm; Genesee Scientific, San Diego, CA, USA). Particles were analyzed for two size fractions: 'large' >210 µm, and 'small' <210 µm. [See Schallenberg et al[25] for further details on sampling POM.]

Sample preparation and lipid extraction

Subsamples from the same sampling site and date were replicated in order to compare values with and without lipid extraction (LE). Prior to SIA, all pteropod samples were rinsed in filtered seawater and weighed. Whilst previous research recommends acidifying thecosome pteropods to remove carbonate content that could otherwise bias stable isotopes results[8], the pressure from the RMT8 sampling method completely stripped entire shells from most *C. pyramidata* samples, and any shell fragments remaining on others were easily removed using forceps. As a result, we did not need to acid-treat our samples prior to further analyses.

Quantitative lipid extractions were conducted using a one-phase methanol/chloroform/Milli-Q water (2:1:0.8 *v*/*v*/*v*; modified from Bligh and Dyer[26], following Phleger *et al*[14]) overnight extraction. This was followed by additional methanol/chloroform/Milli-Q water solution (final ratio of 1:1:0.9 *v*/*v*/*v*) to allow phases to break. The lower lipid phase was removed and the upper mixture was filtered down to a pellet and dried for 24 hr at 60 °C to remove remaining solvents. Non-lipid-extracted ("bulk") samples and POM sample filters were also dried for 24 hr at 60 °C. After oven-drying, individual dry weights of both bulk and lipid-extracted samples were recorded. Small discs were punched from POM filters, and individual pteropods were ground to powder using an agate mortar and pestle, then all were weighed into tin cups in preparation for isotopic analysis.

Stable isotopes analysis

For pteropods, bulk and LE carbon and nitrogen stable isotope ratios were obtained using an automated Elementar vario PYRO cube analyser (Elementar Analysensysteme GmbH, Langenselbold, Germany) coupled with a continuous flow IsoPrime100 isotope ratio mass spectrometer (IsoPrime Ltd, Cheadle Hulme, UK); for POM, samples were analyzed using a

Thermo Scientific Flash 2000HT elemental analyser (Thermo Fisher Scientific, Bremen, Germany) interfaced with a Thermo Fisher Delta V Plus IRMS through a Thermo Fisher Conflo IV. SIA of pteropods was conducted at the Central Science Laboratory (CSL), University of Tasmania (Hobart, Tasmania, Australia), and for POM, at the Australian Nuclear Science and Technology Organisation (ANSTO) in Lucas Heights, Sydney, Australia. Isotopic ratios were expressed in delta (δ) notation and reported as parts per thousand (‰) relative to isotopic reference standards, Vienna Pee Dee Belemnite (for carbon) and atmospheric air (for nitrogen)[4]. To measure instrument stability, analytical precision, drift correction, and linearity performance at CSL, in-laboratory working standards of sulfanilamide, repeated every 6th sample, produced a standard deviation better than ±0.1‰ for both isotope ratios. At ANSTO, POM isotopic data are reported relative to IAEA secondary certified standards, with a standard error of analysis to 1 standard deviation (SD) measured at ± 0.3 ‰. The carbon and nitrogen percentages were converted to atomic C:N ratios (%C/%N x 1.6667). The average standard deviation on triplicate measurements made from randomly selecting pteropod specimens was calculated as 0.15‰ for $\delta^{13}C$ values and 0.19‰ for $\delta^{15}N$ values.

Mathematical normalization and model comparison

The goal of a mathematical normalization model is to assess whether C:N ratios can be used as a predictor of the effect of LE on $\delta^{13}C$ values. This is typically achieved using paired samples (extracted versus untreated) at an individual level, and the relationship between the extraction effect at the individual level ($\Delta\delta^{13}C$) and C:N ratio (prior to LE) is used to 'correct' bulk samples. However, the small body size of our pteropod specimens meant that we were unable to subdivide them to enable direct comparison of the effect of LE within the same specimen. In lieu of testing treatment effects within the same individual, or duplicate aliquots

of pooled individuals possessing potentially high individual variability that would otherwise be lost, we compared average bulk with chemically lipid-extracted $\delta^{13}C$ values of individuals from the same sampling sites. We achieved this through linear modeling the $\delta^{13}C$ values by treatment method to estimate the mean effect of extraction (lm function in R). This simplified approach statistically compares averages and provides an estimate of the effect of LE on $\delta^{13}C$ values.

We compared our corrected $\delta^{13}C$ values with those obtained from published normalization models. We first selected a variation of a standardized mathematical protocol[10] specific to fish tissues that was updated for freshwater zooplankton samples by Kiljunen *et al*[27]. This model estimates the effect of lipids on non-lipid extracted $\delta^{13}C$ values by first calculating a lipid factor (*L*) from bulk C:N ratios (by mass):

$$L = \frac{93}{1 + [0.246 \times (\mathrm{C{:}N_{bulk}}) - 0.775]^{-1}} \quad (1a)$$

which is then applied to the following formula to calculate lipid-extracted $\delta^{13}C$ values ($\delta^{13}C_{LE}$ values):

$$\delta^{13}\mathrm{C_{LE}} = \delta^{13}\mathrm{C_{bulk}} + D \times \left(I + \frac{3.9}{1 + 287/L}\right) \quad (1b)$$

In this model, *D* is the average difference between the $\delta^{13}C$ values of lipid extracted and bulk samples (representing the difference between lipids and proteins) and *I* is a constant. Kiljunen *et al*[27] used values of 7.018 and 0.048 for *D* and *I*, respectively.

The second model was developed by Logan *et al*[28] through comparing chemically- and mathematically-corrected collections of marine fishes and aquatic invertebrates. Like the Kiljunen *et al* model[27], this model assumes a non-linear relationship between bulk C:N ratios (by mass) and $\Delta\delta^{13}C$ values:

$$\delta^{13}C_{LE} = \delta^{13}C_{bulk} + \beta_0 + \beta_1 \ln(C{:}\,N_{bulk}) \quad (2)$$

where the values that we used for β_0 (-2.06) and β_1 (1.91) are parameter estimates specifically applied to all invertebrate species tested[28].

Post *et al*[9] developed the following normalization model based on a strong positive predictive relationship between bulk C:N (by mass) and $\Delta\delta^{13}C$ values, estimated from a large variety of aquatic animal taxa:

$$\delta^{13}C_{LE} = \delta^{13}C_{bulk} - 3.32 + 0.99 \times C{:}\,N_{bulk} \quad (3)$$

The fourth model is based on a mass balance model[29] that was updated by Smyntek *et al*[12] for freshwater zooplankton that incorporates both lipid-extracted and bulk atomic C:N ratios as well as *D* to estimate $\delta^{13}C_{LE}$ values:

$$\delta^{13}C_{LE} = \delta^{13}C_{bulk} + D \times \left[\frac{(C{:}\,N_{bulk} - C{:}\,N_{LE})}{C{:}\,N_{bulk}}\right] \quad (4)$$

where *D* was estimated by Smyntek *et al*[12] to be 6.3; and for $C{:}N_{LE}$, we employed the average values that were empirically obtained for each pteropod species (Table 1).

Finally, we investigated the mass balance-based model developed by Syväranta and Rautio[15] for polar aquatic zooplankton:

$$\delta^{13}C_{LE} = \delta^{13}C_{bulk} + 7.95 \times \left[\frac{(C{:}\,N_{bulk} - 3.8)}{C{:}\,N_{bulk}}\right] \quad (5)$$

where mass $C{:}N_{bulk}$ ratios were used.

We applied these mass balance normalization models to our data and statistically compared the predicted results of each model with ours using a one-way ANOVA (base function in R). This allowed us to compare mean $\delta^{13}C$ values and $\Delta\delta^{13}C$ (the change in $\delta^{13}C$ values from bulk to corrected) values generated from data converted by our average offset value and by the several published normalization models. This was followed by a Tukey's Honest Significant Difference test (base function in R) to determine which published mathematical normalization model produces mean corrected $\delta^{13}C$ and $\Delta\delta^{13}C$ values not significantly different from our calculated average offset value.

Statistical analyses

Data analyses were conducted using the statistical software R, version 3.4.0[30]. We used the Shapiro-Wilk's (function in base R) and Levene's (from the car package, version 3.0-0[31]) tests on bulk and LE isotopic ratios ($\delta^{13}C$ and $\delta^{15}N$ values) to measure normality and homogeneity of variance, respectively. A one-way multivariate analysis of variance (MANOVA; manova function in base R) was used on bulk and LE $\delta^{13}C$ and $\delta^{15}N$ values (as dependent variables) to test for species and treatment effects (as independent variables). Where not otherwise specified, variability is expressed in standard deviations (SD).

To test the effect of lipid correction on isotopic niches, we followed an analytical procedure similar to that of Choy *et al*[32], who measured the effects of lipid extraction on the $\delta^{13}C$ values of beluga whales and prey using niche dispersion metrics as response variables. For each species, we tested the null hypothesis that the mean Euclidean distance between each centroid calculated from each of the niche breadths, estimated from the lipid corrected and unpublished bulk datasets, did not differ from zero. Null distributions were generated by using the residual permutation procedure (RPP) and were then used for comparison with test statistics[33]. The resultant empirical *P* values (the rank percentile of observed differences between groups) were compared with those generated using Hotelling's T^2 test. RPP and Hotelling's T^2 tests were performed following Turner *et al*.[33]

The total and standard ellipse areas (TA and SEA, respectively) of each pteropod species and their potential food sources (POM fractions for *C. pyramidata* and *C. pyramidata* for *C. antarctica* and *S. australis*) were estimated using Stable Isotope Bayesian Ellipses in R (SIBER) version 2.1.3 package[34]. SIBER generates bivariate standard ellipses and convex hulls for isotopic niches. We also measured the proportion of dietary niche overlapping between species using the R package nicheRover version 1.0[35], which produces probable pairwise comparisons between the niche region of each species combination within a Bayesian framework. The niche region of a species is considered to be the 95% probability that a species will be located within isotopic bivariate space, and the niche overlap is defined as the 95% probability that the niche region of one species will overlap with another.

Results

Effect of lipid-extraction on isotopic ratios

Thirty-eight pteropod individuals representing three species and sampled from 12 sites were lipid-extracted (LE) (Table S1, supporting information). Significant increases for all species in $\delta^{13}C$ values (mean $\Delta = 2.43 \pm 0.2‰$) and in $\delta^{15}N$ values (mean $\Delta = 1.11 \pm 0.8‰$) occurred in samples with lipids removed prior to SIA compared with untreated ($n = 61$) samples from the same sampling sites (MANOVA: Wilk's λ: $F_{2,74} = 105.1$, $P < 0.001$; Table S2, supporting information). Multivariate analysis also detected significant differences in mean $\delta^{13}C$ and $\delta^{15}N$ values between species (MANOVA, Wilk's λ: species: $F_{4,148} = 28.6$, $P < 0.001$; Table S2, supporting information).

The bulk C:N ratios for all species ranged from 3.6 to 8.0 (average 4.6 ± 1.3), which was more variable than the LE C:N ratios that ranged from 3.2 to 4.1 (Figure 1A, Table 1). The largest magnitude of increase in $\delta^{13}C$ values (LE – bulk) was detected in the gymnosome pteropods (*S. australis*: +4.0‰; *C. antarctica*: +4.5‰; Table S1, supporting information). The average (by site) bulk C:N ratio for all pteropods was 5.0 ± 1.5, with a range that varied between and within species (3.7 – 7.4); however, lipid extracted samples possessed a more reduced intersample range (3.2 – 4.1), along with a much lower average C:N ratio of 3.4 ± 0.2 (Table S1, supporting information). The largest variation in the bulk C:N ratio in a given species was measured in *C. pyramidata* (3.7 – 4.4). *C. antarctica* was the only species to have a mean bulk C:N ratio >7.0, whereas *C. pyramidata* had the smallest mean bulk C:N ratio (4.0 ± 0.2).

Treatment also affected the carbon and nitrogen contents, whereby the range of both % carbon and % nitrogen were reduced relative to untreated values, while also demonstrating a

net overall increase in both (Figure 1B). The net effect of reduced C:N ratios from extraction was a result of a relatively greater effect on % nitrogen (Figures 2A and 2B).

Lipid normalization model comparison and selection

The results from our modelling approach ($\delta^{13}C$ ~ treatment) determined that 63% of the variation in $\delta^{13}C$ values was accounted for by extraction treatment alone (Figure 3). Multiple pairwise comparisons made between each model- and the offset-corrected mean $\delta^{13}C$ value revealed statistically significant differences between datasets except for the models developed by Kiljunen *et al*[27] and Syväranta and Rautio[15] (Figure 4; Table S3, supporting information). When comparisons were made on the average corrected change from bulk to corrected values with our average offset value, all values were significantly different except for those from the model by Kiljunen *et al*[27].

Effect of lipids on niche dispersion metrics

The total area was larger for bulk than for lipid-extracted samples, whereas lipid-extracted SEAs and SEA_cs were larger than those measured for the bulk dataset (Figure 5, Table 2). RPP and Hotelling's T^2 tests revealed that the Euclidean distances between centroids of isotopic niches measured before and after lipid correction differed significantly from zero for all species (*C. pyramidata* distance = 2.38; $p = 0.001$; Hotelling's $T^2 = 99.81$; $P < 0.0001$; *C. antarctica* distance = 2.84; $p = 0.001$; Hotelling's $T^2 = 97.3$; $P < 0.001$; *S. australis* distance = 2.61; $p = 0.001$; Hotelling's $T^2 = 30.41$; $P < 0.001$). This means that there were statistically significant differences in the distances between centroids calculated for treated and untreated pteropods of each species.

The total and standard ellipse areas (TA and SEA, respectively) for both large (>210 µm) and small (<210 µm)-fractionated POM samples were calculated (small fraction: TA = 1.86‰^{2},

SEA = 1.22‰^{2}; large fraction: TA = 2.18‰^{2}, SEA = 1.47‰^{2}), and subsequently combined as one potential food source to compare niche overlaps with the SEAs calculated for bulk and lipid-corrected (LC) *C. pyramidata* (Figure 6A). The 95% probabilistic niche area overlaps of bulk and POM standard ellipses were greater than the overlaps between the LC and POM standard area ellipses of both bulk and LC *C. pyramidata* (bulk + POM: 0.34%; LC + POM: 0.04%). However, when the SEAs of *S. australis* were compared with that of its potential food source, represented by bulk *C. pyramidata*, overlapping with corrected samples was higher than with bulk samples (Figure 6B; bulk + POM, 29.0%; LC + POM, 90.7%).

Comparisons made between pteropod data corrected using our average offset value and data corrected using normalization models revealed no significant differences for each niche dispersion metric (Figure 7; Table S4, supporting information), including TA ($F(3, 7) = 0.05$, $p = 0.985$) and SEA ($F(3, 7) = 0.11$, $p = 0.949$).

Discussion

Chemical lipid extraction vs bulk samples

Lipid extraction consistently produced statistically significant differences in $\delta^{13}C$ values for all pteropod species and sampling sites examined, with a 2.4 ± 0.2‰ average increase compared with bulk values. When we modelled the mean effect of treatment on $\delta^{13}C$ values, 63% of the variation in $\delta^{13}C$ values was accounted for by lipid extraction alone. We also found that, for all species combined, the lipid extracted samples had significantly higher $\delta^{15}N$ values, by 1.1 ± 0.8 ‰, than bulk samples. This difference in $\delta^{15}N$ values is not uncommon in marine species and is well within the variable offset range (-2.3 to +1.8 ‰) reported by Svensson *et al*[36] for several fish and invertebrates. This is probably a function of variable loss

of solvent-soluble nitrogenous materials and proteins depleted in ^{15}N relative to bulk tissues, such as the amino acid serine, from lipids[36,37]. This was also demonstrated by the net increase in % nitrogen in all species that may have significantly affected the decreasing C:N ratios.

We observed an increase in variability within extracted versus untreated bulk $\delta^{13}C$ values, which some previous studies have reported as a function of removing lipids [19,38,39]. We did detect a decrease in variance calculated in the C:N ratios of lipid-extracted relative to bulk samples. C:N ratios are commonly used as a proxy for lipid content, with high C:N ratios assumed to indicate higher lipid content[9,27]. They are calculated from % carbon and nitrogen values, which also each showed decreased variances after lipid extraction. The Bligh and Dyer method[26] employed in our analysis produced C:N ratios ranging from 3.2 to 4.1 (average = 3.4 ± 0.2) from all species combined, in comparison with the higher variance measured in bulk C:N ratios, ranging from 3.6 to 8.0 (average = 4.6 ± 1.3). Taken separately, the gymnosome *C. antarctica* yielded the highest average bulk and lipid-extracted C:N ratios, whereas the other gymnosome analyzed, *S. australis*, showed the greatest difference in $\delta^{13}C$ values between treatments. These results could point to an incomplete delipidation for *C. antarctica*, particularly given that it is less clear to see the net increase in % carbon from these extracted samples than for *C. pyramidata* and *S. australis*. They may also suggest a potentially strong species-specific response to lipid extraction, consequently pointing to greater difficulty when inferring a strong relationship between bulk C:N ratios and lipid content. This conclusion is further corroborated by similar responses to lipid extraction and acidification of many Arctic and sub-Arctic marine zooplankton assemblages[8].
Unfortunately, due to the low sample sizes of the gymnosomes, we were unable to model a species-specific lipid normalization in order to determine whether C:N ratios can appropriately serve as a proxy for lipid content at species level. Choy *et al*[32] found no

relationship between bulk C:N ratios and $\Delta\delta^{13}C$ values in isopod and shrimp species, and questioned the utility of approximating C:N ratios for % lipid in many species of marine invertebrates and fish[8,27]. Future directed studies measuring species-specific proportion of lipids (measured in dry weight) and its relationship to untreated C:N ratios of pteropods are encouraged, with, wherever possible, larger sample sizes.

Normalization model selection and the effect of lipid correction on niche dispersion metrics

The normalization model of Kiljunen *et al*[27] most accurately predicted extracted $\delta^{13}C$ values for all pteropod species combined, and can provide a suitable alternative to chemical lipid extraction in pteropod-based isotopic research. This outcome is surprising, considering that model efficiency tests in other studies have shown this model to be appropriate for correcting $\delta^{13}C$ values from high lipid content tissues such as fish liver[40]. Although their own model provided the best fit (R^2 = 0.86) for their subarctic and boreal lakes zooplankton data, Sÿvaranta and Rautio[15] found that model of Kiljunen et al.[27] fitted their data more closely (R^2 = 0.65) than any other normalization model tested. The model by Sÿvaranta and Rautio[15] also produced an average $\delta^{13}C$ value closely resembling the average value calculated by data corrected via our offset value. This, however, is not surprising considering that this model was developed for polar zooplankton. Our results indicate minor differences in the effect of mathematical normalization model selection on the niche dispersion metrics of standard ellipses and total areas, although not sufficient to distort the interpretation of overall trophic structure. Due to our relatively small sample sizes, particularly when accounting for species separately, we understand that total and standard ellipse areas can be inaccurate when used as a proxy for isotopic niche areas[41], and thus exercise considerable caution when interpreting these results. Syväranta et al.[41] generated simulations of varying sample sizes from among

two fish populations and found that sizes greater than 30 were still insufficient to improve the uncertainty surrounding niche analyses, particularly when using total area estimations. Further research is required to determine the appropriate minimum samples sizes needed to perform robust estimations of trophic niche breadth in pteropods using standard ellipse and total areas.

Murry *et al*[19] found that, relative to bulk samples, lipid extraction of tissues from an entire fish community altered the $\delta^{13}C$ and $\delta^{15}N$ values to significantly shift the position of the entire food web, rather than to induce smaller scale effects such as relative trophic positions among species. Since our normalization model was based on all species combined, applying this to correct bulk tissues of all species produced a similar result, wherein the entire community significantly shifted without affecting the positioning of each species relative to other species. We also calculated species-specific standard area ellipses and centroid locations separately for both lipid-corrected and bulk $\delta^{13}C$ values to determine the existence of isotopic niche overlapping with potential food sources, and whether the magnitude of overlapping could bias interpretations of resource partitioning. Higher within-sample variability was measured in bulk versus lipid-extracted and lipid-corrected $\delta^{13}C$ values, particularly in total area measurements, which was similar to the results Choy *et al*[32] found in a number of polar organisms and tissues. Unlike Murry *et al*[19], Choy *et al*[32] did not detect community-level change with respect to differences in niche dispersion metrics, and pointed at the need to examine community-based niche dynamics at the species level. We measured varying degrees of overlapping between the standard area ellipses of pteropod species and their potential food sources, with bulk *C. pyramidata* displaying higher overlapping with POM than corrected *C. pyramidata*, and corrected gymnosome species (*C. antarctica* and *S. australis*) displaying higher overlapping with *C. pyramidata* than bulk gymnosome species

niches. This variability highlights the need to correct for lipids in untreated samples, and shows how ignoring this procedure can affect interpretations of trophic relationships between and among species assemblages, particularly if species are directly connected through ecologically significant predator-prey interactions or competition.

Conclusions

Given that we observed variability in niche overlapping measured between untreated and mathematically corrected isotopic ratios of pteropods with their diet preferences, we strongly encourage the incorporation of lipid correction within the development of research protocols involving pteropod-focused niche breadth analyses. Without lipid correction of $\delta^{13}C$ values, be it through chemical extraction or *a posteriori* mathematical normalization, the magnitude of species interactions and resource partitioning can be over- or underestimated within polar pteropod assemblages. When analyzing samples possessing similar untreated C:N ratios to those observed here (3.5-8.0), and chemical extraction of a subset is not an option, we recommend selecting models calibrated to similar ratios and organisms (eg.[15,27]). We found high species-specific variability in relationships between untreated C:N ratios and $\Delta\delta^{13}C$ values, and thus strongly encourage incorporation of larger sample sizes, although it may be challenging for less common species, such as polar gymnosomes. Lipid extraction also affected $\delta^{15}N$ values, and we consequently support analytical treatments of $\delta^{13}C$ and $\delta^{15}N$ values separately[32,42]. We advocate using well-developed research protocols, as doing so will provide further information about a taxonomic group particularly sensitive to anthropogenic change to ocean chemistry.

Acknowledgements

First, we thank the anonymous reviewers for their valuable editorial comments that greatly improved this manuscript. We would like to acknowledge the valuable support from the master and crew as well as scientific and technical support teams aboard the RV *Aurora Australis*. We are also grateful to Di Davies (CSIRO) and Barbara Gallagher (ANSTO) for POM sampling and isotopic analyses, respectively, as well as Christine Cook, Christian Dietz, and Thomas Rodemann for assistance with mass balance and running pteropod isotopic analyses at CSL. We thank Jake Wallis for assistance with assembling and assistance with laboratory setup for lipid extractions. This work was financially supported by Australian Antarctic Science grants 4331 and 4344, the Holsworth Wildlife Research Endowment (grant 109804) – Equity Trustees Charitable Foundation and the Ecological Society of Australia, and an ANSTO United Uranium Scholarship (grant 10043). RT was supported by the RJL Hawke Postdoctoral Fellowship, and this work was further supported by the Australian Government's Business Cooperative Research Centres Programme through the Antarctic Climate and Ecosystems Cooperative Research Centre (ACE CRC).

References

1. Newsome SD, Rio, Martinez del Rio C, Bearhop S, Phillips DL. A Niche for Isotope Ecology. *Front Ecol Environ*. 2007;5(8):429-436. doi:10.1890/060150.01.
2. Layman CA, Quattrochi JP, Peyer CM, Allgeier JE. Niche width collapse in a resilient top predator following ecosystem fragmentation. *Ecol Lett*. 2007;10(10):937-944. doi:10.1111/j.1461-0248.2007.01087.x.
3. Fry B. *Stable Isotope Ecology*. 1st ed. New York: Springer-Verlag; 2006. doi:10.1007/0-387-33745-8.
4. DeNiro MJ, Epstein S. Influence of diet on the distribution of carbon isotopes in animals. *Geochim Cosmochim Acta*. 1978;42(5):495-506. doi:10.1016/0016-7037(78)90199-0.
5. Peterson B, Fry B. Stable isotopes in ecosystem studies. *Ann Rev Ecol Syst*. 1987;18(1):293-320. doi:10.1146/annurev.es.18.110187.001453.
6. Minagawa M, Wada E. Stepwise enrichment of ^{15}N along food chains: Further evidence and the relation between $\delta^{15}N$ and animal age. *Geochim Cosmochim Acta*. 1984;48(5):1135-1140. doi:10.1016/0016-7037(84)90204-7.
7. Marcus L, Virtue P, Nichols PD, Meekan MG, Pethybridge H. Effects of sample treatment on the analysis of stable isotopes of carbon and nitrogen in zooplankton, micronekton and a filter-feeding shark. *Mar Biol*. 2017;164(6):124. doi:10.1007/s00227-017-3153-6.
8. Pomerleau C, Winkler G, Sastri A, Nelson RJ, Williams WJ. The effect of acidification and the combined effects of acidification/lipid extraction on carbon stable isotope ratios for sub-arctic and arctic marine zooplankton species. *Polar Biol*. 2014;37(10):1541-1548. doi:10.1007/s00300-014-1540-8.
9. Post DM, Layman CA, Arrington DA, Takimoto G, Quattrochi J, Montaña CG. Getting to the fat of the matter: Models, methods and assumptions for dealing with lipids in stable isotope analyses. *Oecologia*. 2007;152(1):179-189. doi:10.1007/s00442-006-0630-x.
10. McConnaughey T, McRoy CP. Food-Web structure and the fractionation of Carbon isotopes in the bering sea. *Mar Biol*. 1979;53(3):257-262. doi:10.1007/BF00952434.
11. Kattner G, Hagen W, Graeve M, Albers C. Exceptional lipids and fatty acids in the pteropod *Clione limacina* (Gastropoda) from both polar oceans. *Mar Chem*. 1998;61(3-4):219-228. doi:10.1016/S0304-4203(98)00013-9.
12. Smyntek PM, Teece MA, Schulz KL, Thackeray SJ. A standard protocol for stable isotope analysis of zooplankton in aquatic food web research using mass balance correction models. *Limnol Oceanogr*. 2007;52(5):2135-2146.

doi:10.4319/lo.2007.52.5.2135.

13. Phleger CF, Nelson MM, Mooney BD, Nichols PD. Interannual variations in the lipids of the Antarctic pteropods *Clione limacina* and *Clio pyramidata*. *Comp Biochem Physiol - B Biochem Mol Biol*. 2001;128(3):553-564. doi:10.1016/S1096-4959(00)00356-0.

14. Phleger CF, Nichols PD, Virtue P. Lipids and buoyancy in Southern Ocean pteropods. *Lipids*. 1997;32(10):1093-1100. doi:10.1007/s11745-997-0141-x.

15. Syväranta J, Rautio M. Zooplankton, lipids and stable isotopes: importance of seasonal, latitudinal, and taxonomic differences. *Can J Fish Aquat Sci*. 2010;67(November):1721-1729. doi:10.1139/F10-091.

16. Conover RJ, Lalli CM. Feeding and growth in *Clione limacina* (Phipps), a pteropod mollusc. *J Exp Mar Bio Ecol*. 1972;9(3):279-302. doi:10.1016/0022-0981(72)90038-X.

17. Hunt BPV, Pakhomov EA, Hosie GW, Siegel V, Ward P, Bernard K. Pteropods in Southern Ocean ecosystems. *Prog Oceanogr*. 2008;78(3):193-221. doi:10.1016/j.pocean.2008.06.001.

18. Hussey NE, MacNeil MA, McMeans BC, et al. Rescaling the trophic structure of marine food webs. *Ecol Lett*. 2014;17(2):239-250. doi:10.1111/ele.12226.

19. Murry BA, Farrell JM, Teece MA, Smyntek PM. Effect of lipid extraction on the interpretation of fish community trophic relationships determined by stable carbon and nitrogen isotopes. *Can J Fish Aquat Sci*. 2006;63(10):2167-2172. doi:10.1139/f06-116.

20. Jia Z, Swadling KM, Meiners KM, Kawaguchi S, Virtue P. The zooplankton food web under East Antarctic pack ice – A stable isotope study. *Deep Res Part II Top Stud Oceanogr*. 2016;131:189-202. doi:10.1016/j.dsr2.2015.10.010.

21. Manno C, Giglio F, Stowasser G, Fielding S, Enderlein P, Tarling GA. Threatened species drive the strength of the carbonate pump in the northern Scotia Sea. *Nat Commun*. 2018;(2018):1-7. doi:10.1038/s41467-018-07088-y.

22. Lalli CM, Gilmer RW. *Pelagic Snails: The Biology of Holoplanktonic Gastropod Mollusks*. Stanford University Press; 1989. https://books.google.com.au/books/about/Pelagic_Snails.html?id=yIAfwz5cxPMC. Accessed September 24, 2017.

23. Orr JC, Fabry VJ, Aumont O, et al. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature*. 2005;437(7059):681-686. doi:10.1038/nature04095.

24. Manno C, Bednaršek N, Tarling GA, et al. Shelled pteropods in peril: Assessing vulnerability in a high CO_2 ocean. *Earth-Science Rev*. 2017;169(August 2016):132-145. doi:10.1016/j.earscirev.2017.04.005.

25. Schallenberg C, Bestley S, Klocker A, et al. Sustained Upwelling of Subsurface Iron Supplies Seasonally Persistent Phytoplankton Blooms Around the Southern Kerguelen Plateau, Southern Ocean. *J Geophys Res Ocean*. 2018;123(8):5986-6003. doi:10.1029/2018JC013932.

26. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*. 1959;37(8):911-917. doi:dx.doi.org/10,1139/cjm2014-0700.

27. Kiljunen M, Grey J, Sinisalo T, Harrod C, Immonen H, Jones RI. A revised model for lipid-normalizing $\delta^{13}C$ values from aquatic organisms, with implications for isotope mixing models. *J Appl Ecol*. 2006;43(6):1213-1222. doi:10.1111/j.1365-2664.2006.01224.x.

28. Logan JM, Jardine TD, Miller TJ, Bunn SE, Cunjak RA, Lutcavage ME. Lipid corrections in carbon and nitrogen stable isotope analyses: Comparison of chemical extraction and modelling methods. *J Anim Ecol*. 2008;77(4):838-846. doi:10.1111/j.1365-2656.2008.01394.x.

29. Fry B, Baltz DM, Benfield MC, et al. Stable isotope indicators of movement and residency for brown shrimp (*Farfantepenaeus aztecus*) in coastal Louisiana marshscapes. *Estuaries*. 2003;26(1):82-97. doi:10.1007/BF02691696.

30. Team RC. R: A language and environment for statistical computing. 2014. http://www.r-project.org/.

31. Fox J, Weisberg S. *An R Companion to Applied Regression*. Second. Thousand Oaks, CA, USA: Sage Publications; 2011.

32. Choy ES, Roth JD, Loseto LL. Lipid removal and acidification affect nitrogen and carbon stable isotope ratios of beluga whales (*Delphinapterus leucas*) and their potential prey species in the Beaufort Sea ecosystem. *Mar Biol*. 2016;163(10):220. doi:10.1007/s00227-016-2992-x.

33. Turner TF, Collyer ML, Krabbenhoft TJ. A general hypothesis-testing framework for stable isotope ratios in ecological studies. *Ecology*. 2010;91(8):2227-2233. doi:10.1890/09-1454.1.

34. Jackson AL, Inger R, Parnell AC, Bearhop S. Comparing isotopic niche widths among and within communities: SIBER - Stable Isotope Bayesian Ellipses in R. *J Anim Ecol*. 2011;80(3):595-602. doi:10.1111/j.1365-2656.2011.01806.x.

35. Swanson HK, Lysy M, Power M, Stasko AD, Johnson JD, Reist JD. A new probabilistic method for quantifying n-dimensional ecological niches and niche overlap. *Ecology*. 2014;96(2):318-324. doi:10.1890/14-0235.1.

36. Svensson E, Schouten S, Hopmans EC, Middelburg JJ, Damsté JSS. Factors controlling the stable nitrogen isotopic composition ($\delta^{15}N$) of Lipids in Marine Animals. *PLoS One*. 2016;11(1):1-12. doi:10.1371/journal.pone.0146321.

37. Sweeting CJ, Polunin NVC, Jennings S. Effects of chemical lipid extraction and arithmetic lipid correction on stable isotope ratios of fish tissues. *Rapid Commun Mass Spectrom*. 2006;20(4):595-601. doi:10.1002/rcm.2347.

38. DeNiro MJ, Epstein S. Mechanism of carbon isotope fractionation associated with lipid synthesis. *Science (80-)*. 1977;197(4300):261-263. doi:10.1126/science.327543.

39. Pinnegar JK, Polunin NVC. Differential fractionation of $\delta^{13}C$ and $\delta^{15}N$ among fish tissues: Implications for the study of trophic interactions. *Funct Ecol*. 1999;13(2):225-231. doi:10.1046/j.1365-2435.1999.00301.x.

40. Skinner MM, Martin AA, Moore BC. Is lipid correction necessary in the stable isotope analysis of fish tissues? *Rapid Commun Mass Spectrom*. 2016;30(7):881-889. doi:10.1002/rcm.7480.

41. Syväranta J, Lensu A, Marjomäki TJ, Oksanen S, Jones RI. An Empirical Evaluation of the Utility of Convex Hull and Standard Ellipse Areas for Assessing Population Niche Widths from Stable Isotope Data. Getz WM, ed. *PLoS One*. 2013;8(2):e56094. doi:10.1371/journal.pone.0056094.

42. Ryan C, McHugh B, Trueman CN, Harrod C, Berrow SD, O'Connor I. Accounting for the effects of lipids in stable isotope ($\delta^{13}C$ and $\delta^{15}N$ values) analysis of skin and blubber of balaenopterid whales. *Rapid Commun Mass Spectrom*. 2012;26(23):2745-2754. doi:10.1002/rcm.6394.

Table 1 Species-specific average (± SD) bulk, lipid-extracted (LE), and lipid-corrected (LC) $\delta^{13}C$ values and bulk and LE C:N ratios. LC values were obtained using our calculated average offset value of $\Delta\delta^{13}C = 2.4 \pm 0.2‰$ from our simplified modelling approach.

Species	Bulk $\delta^{13}C$	LE $\delta^{13}C$	LC $\delta^{13}C$	C:N (bulk)	C:N (LE)
C. pyramidata	-28.1 ± 0.9	-25.4 ± 0.6	-25.7 ± 0.9	3.9 ± 0.3	3.3 ± 0.1
C. antarctica	-28.3 ± 1.0	-26.6 ± 1.2	-23.7 ± 0.8	6.4 ± 1.4	3.8 ± 0.4
S. australis	-27.9 ± 1.0	-24.0	-25.3 ± 0.9	5.1 ± 0.4	3.3

Table 2 Total area (TA), standard ellipse area (SEA) and SEA corrected for small sample sizes (SEA_c) for all pteropod species combined.

	TA ($‰^2$)	SEA ($‰^2$)	SEAc ($‰^2$)	*N*
Bulk	10.38	2.29	2.33	61
Lipid-extracted	9.35	2.35	2.41	38

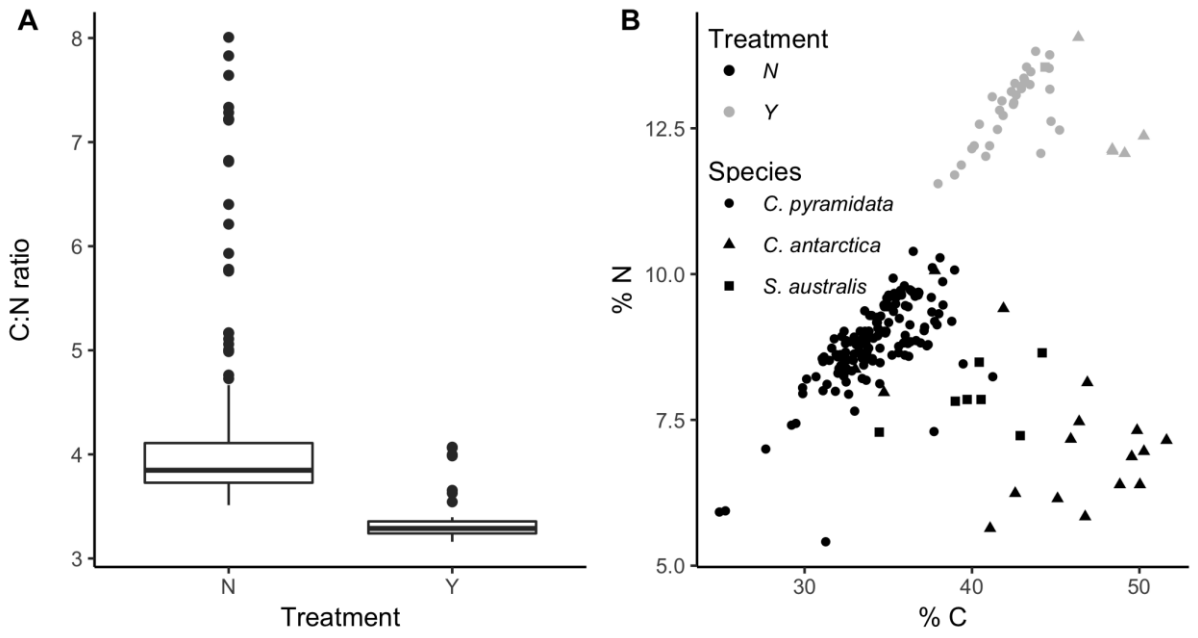

Figure 1. (A) Relationship between treatment (N = bulk, Y = lipid-extracted) and C:N ratio; (B) relationship between percent carbon and percent nitrogen for both treatments (N = bulk (black), Y = lipid-extracted (grey)) and grouped by species (circle = *C. pyramidata*, triangle = *C. antarctica*, square = *S. australis*).

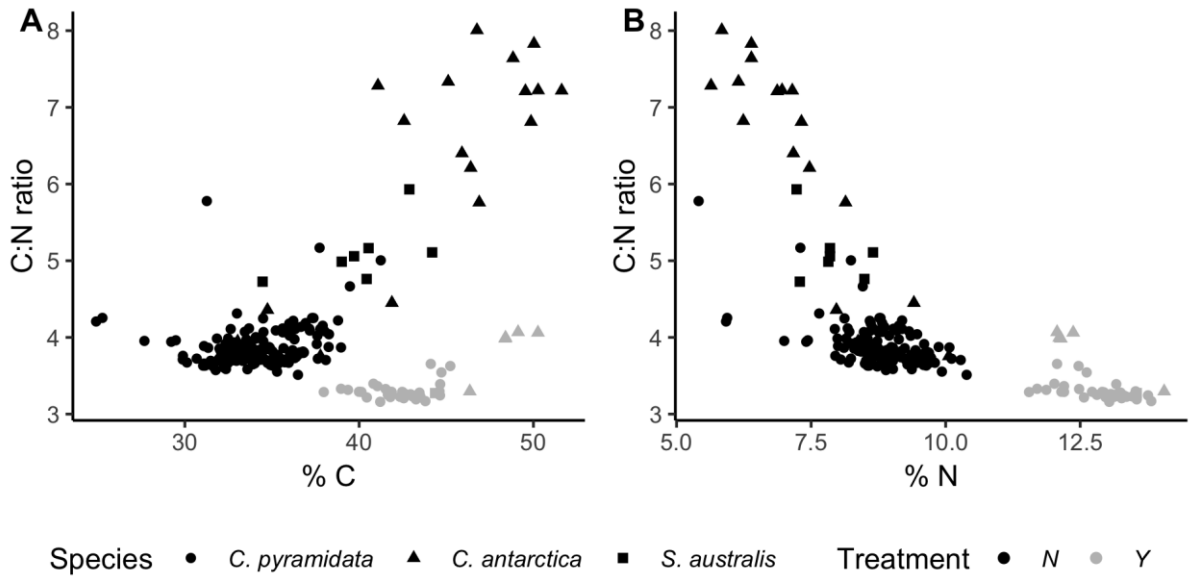

Figure 2. Relationship between (A) % carbon, and (B) % nitrogen and C:N ratio for each treatment (N = bulk (black), Y = lipid-extracted (grey)) and species (circle = *C. pyramidata*, triangle = *C. antarctica*, square = *S. australis*).

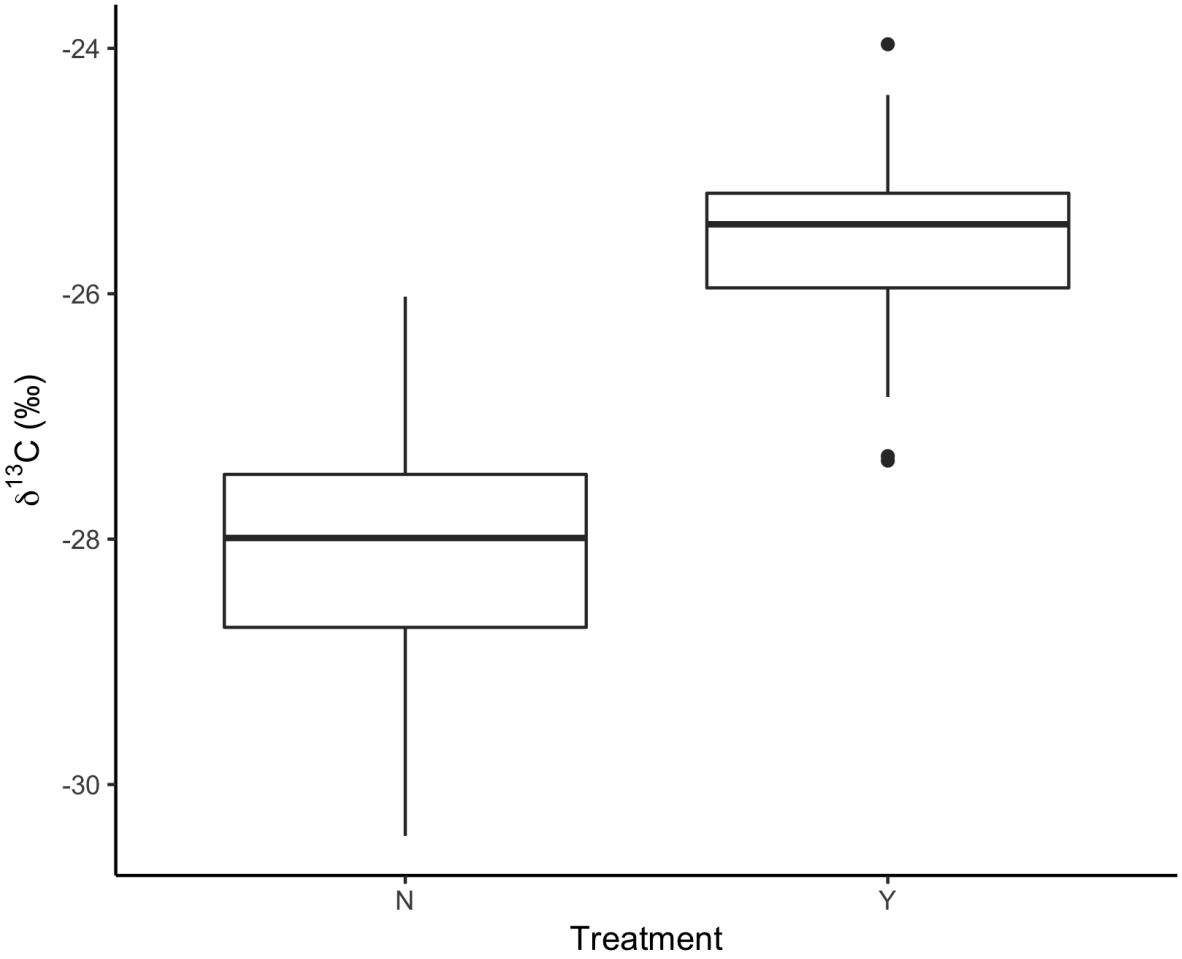

Figure 3. Relationship between each treatment (N = bulk, Y = lipid-extracted) and $\delta^{13}C$ values.

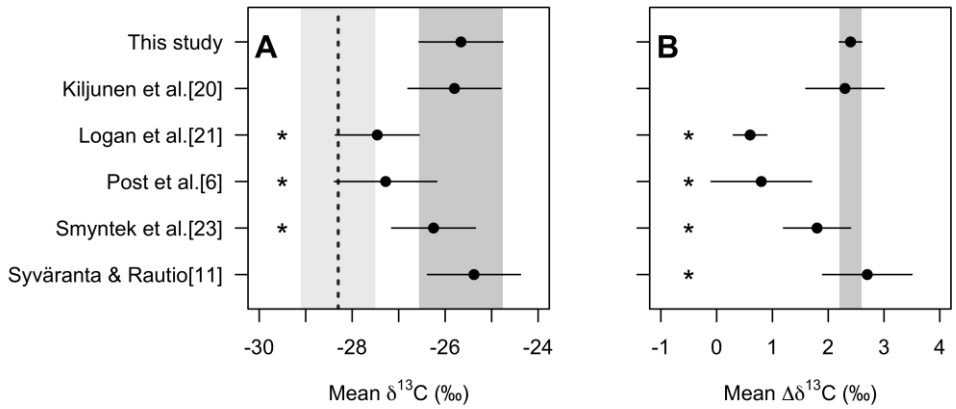

Figure 4. Summary results of multiple pairwise comparisons made between (A) mean $\delta^{13}C$ values (black dots) derived from correcting $\delta^{13}C_{bulk}$ values by each model with the mean $\delta^{13}C_{corrected}$ value derived from our offset value ("This study"); and (B) $\Delta\delta^{13}C$ values ($\delta^{13}C_{bulk}$ - $\delta^{13}C_{corrected}$) derived from each model with the $\Delta\delta^{13}C$ values obtained from our offset value. Dark grey vertical bands denote standard deviations of the mean values obtained from using our offset value to correct bulk. The mean $\delta^{13}C_{bulk}$ values in (A) are denoted by the dashed vertical lines with the standard deviations of the mean shown by the light grey vertical bands. Horizontal error bars refer to standard error of the means. Asterisks refer to values that are significantly different from the means obtained from this study.

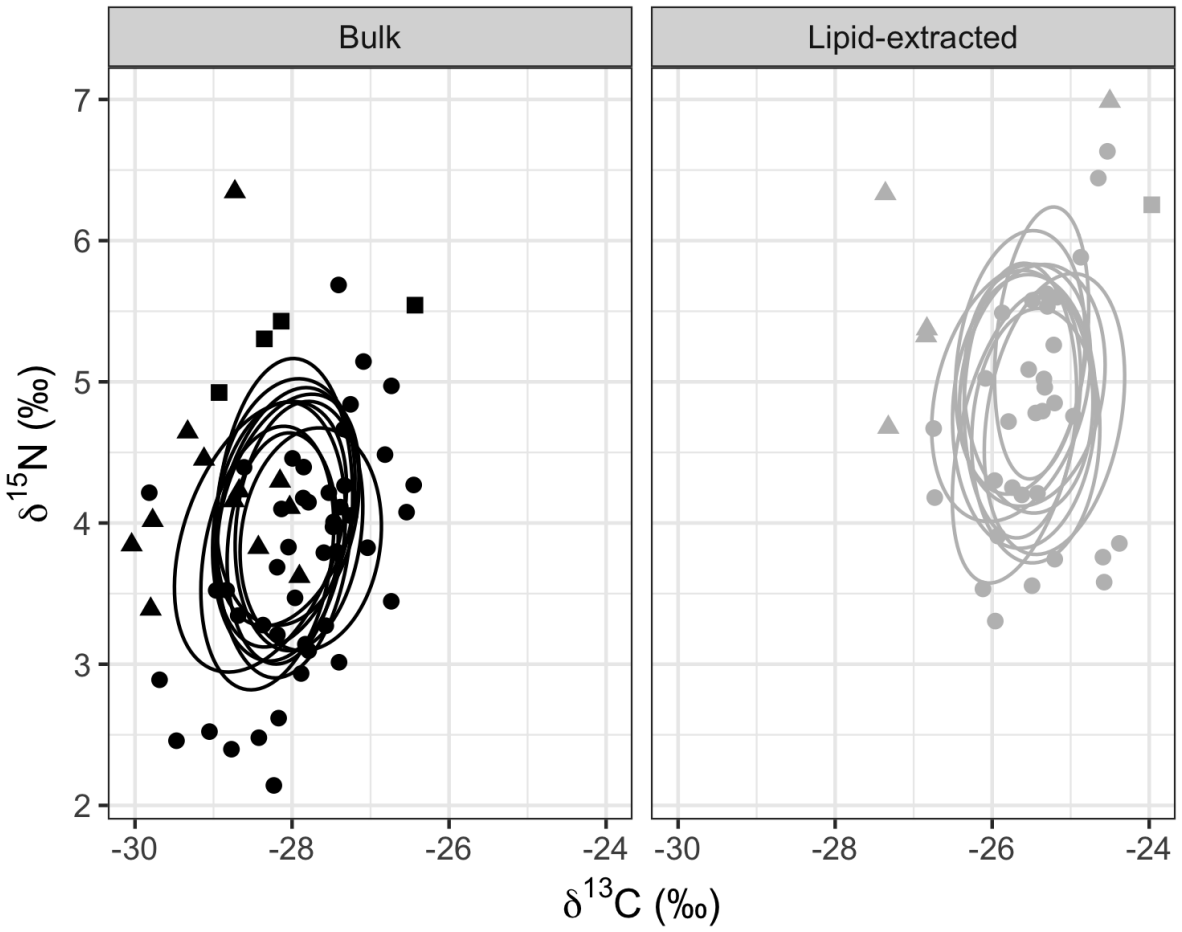

Figure 5. Bulk (black) versus LE (grey) $\delta^{13}C$ and bulk $\delta^{15}N$ values for *C. pyramidata* (circles), *C. antarctica* (triangles), and *S. australis* (squares). Ellipses represent ten random elliptical projections of trophic niche space and contain ~40 % of the data.

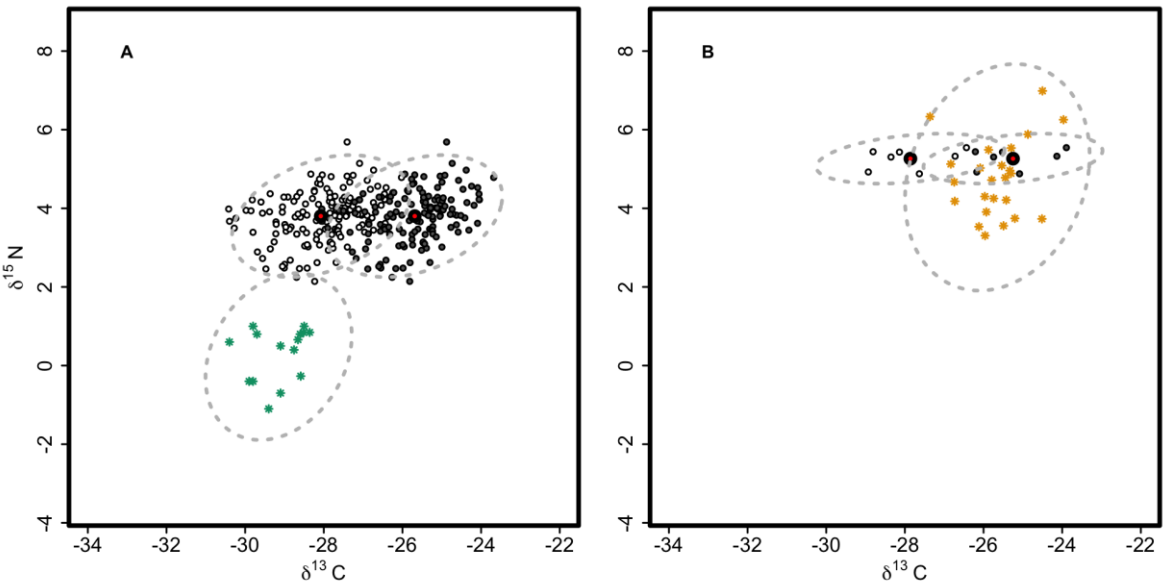

Figure 6. Isotopic niches including standard ellipses (grey dashed line) of pteropod species applied to bulk (white circles) and lipid-corrected (LC) $\delta^{13}C$ values (dark grey circles) for (A), *C. pyramidata*, and (B), *S. australis*. Red points are centroid values. Green and yellow stars represent standard area ellipse of marine POM and lipid extracted *C. pyramidata*, respectively.

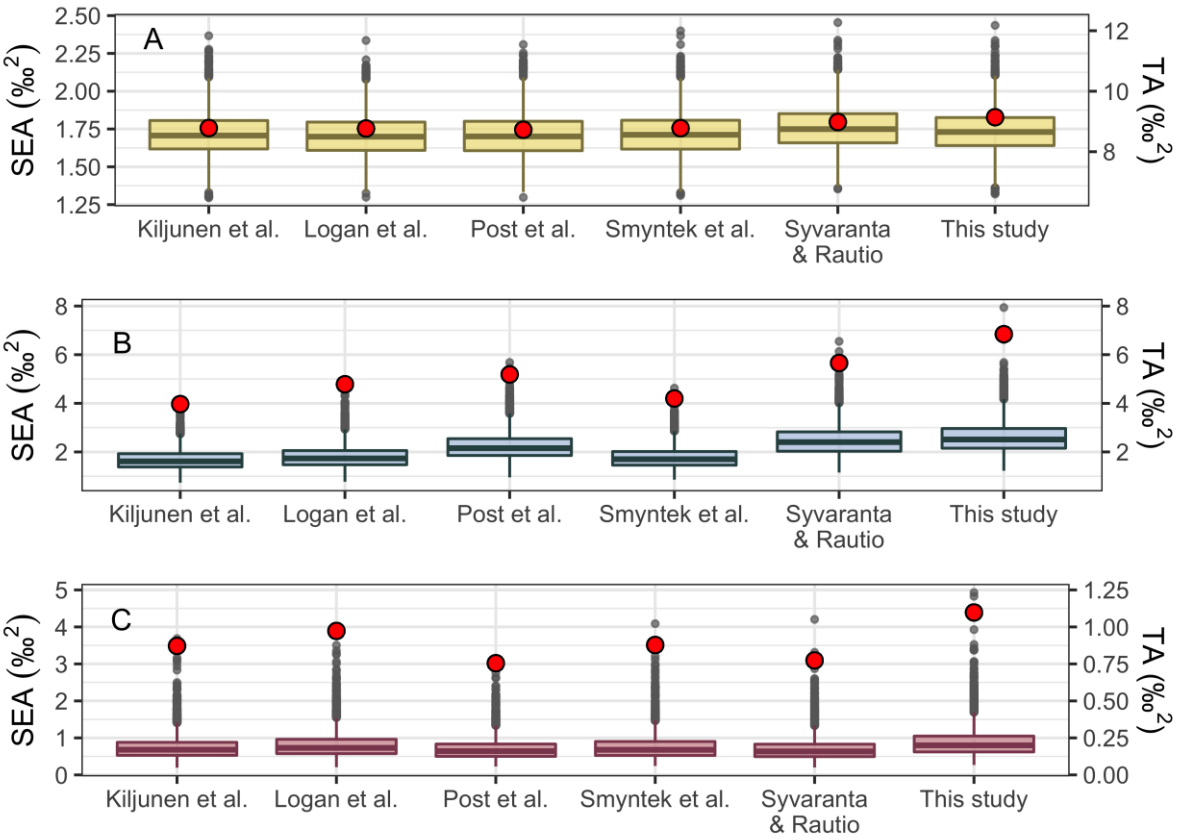

Figure 7. Differences in standard area ellipses (SEA) and total area of the convex hull (red circles) between data that was not lipid-corrected (bulk), and corrected using each normalization model for (A) *C. pyramidata*, (B) *C. antarctica*, and (C) *S. australis*. The bottom and top of the box are the 25th and 75th percentiles, respectively; the horizontal line bisecting the box is the 50th percentile. The whiskers span the highest to the lowest value observations; outliers (grey circles) are the observations plotted outside of this range.